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(CSF-1) and Tumor-Associated Macrophages in Breast Tumor  
Malignancy

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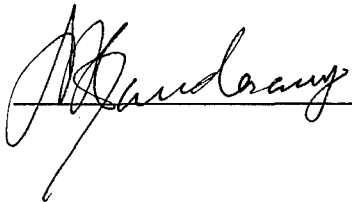
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13. ABSTRACT (Maximum 200 words) The aim of this proposal is to determine the causal role for the mononuclear phagocytic growth factor, CSF-1, overexpressed in human breast cancer, as well as for tumor-associated macrophages in tumor progression. Our recent studies indicate that CSF-1 and CSF-1R play important roles in mammary gland development because CSF-1 nullizygous mice ( <i>Csf1<sup>op</sup>/Csf1<sup>op</sup></i> ) display a failure of branching morphogenesis associated with an almost complete absence of macrophages in <i>Csf1<sup>op</sup>/Csf1<sup>op</sup></i> mice at mid pregnancy. We tested the hypothesis that the potentiation of ductal cell growth by CSF-1 and/or CSF-1 recruited macrophages may be similar in both normal and malignant processes by analyzing <i>in vivo</i> the development of mammary gland in the presence (+/ <i>Csf1<sup>op</sup></i> mice) or absence ( <i>Csf1<sup>op</sup>/Csf1<sup>op</sup></i> mice) of CSF-1. One of the tumor <i>Csf1<sup>op</sup>/Csf1<sup>op</sup></i> mammary mouse model in the transgenic MMTV-PyV middle T background showed that in absence of CSF-1, the tumor progression is delayed and correlated to the lack of macrophage, which are abundant around the carcinoma cells in +/ <i>Csf1<sup>op</sup></i> mice. In parallel, mammary gland postnatal development study demonstrates that CSF-1 is required for epithelial ductal outgrowth acting through the CSF-1R expressed on epithelial cells and macrophages in order to recruit them around the terminal end buds. These studies, together with ongoing analyses of mammary tumor-susceptible <i>Csf1<sup>op</sup>/Csf1<sup>op</sup></i> mice, will offer insight into the autocrine role of tumor-secreted CSF-1 and its paracrine effect on macrophages, emphasizing the importance of stromal/epithelial cell interactions in normal and malignant mammary gland development.					
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## INTRODUCTION

Colony stimulating factor-1 (CSF-1) is a circulating homodimeric growth factor that regulates the survival, proliferation, motility and differentiation of cells of the mononuclear phagocytic lineage (1,2). The effects of CSF-1 are mediated by a high affinity cell surface receptor (CSF-1R) encoded by the *c-fms* proto-oncogene, which is a class III transmembrane tyrosine kinase (3). Confirmation of the roles for CSF-1 in the regulation of mononuclear phagocytes comes from studies with a CSF-1 null mutant mouse, osteopetrotic (*Csfmop*). This mouse contains a thymidine insertion in the 5' end of the CSF-1 gene, which results in a translational frameshift and premature termination of translation. Therefore, homozygous mutant mice are completely devoid of CSF-1 (4,5). Phenotypically the homozygous mutants are severely depleted of cells of the mononuclear phagocytic lineage including macrophages and osteoclasts, confirming the central role of CSF-1 in the regulation of these cells (4,6,7). The deficiency in osteoclasts results in a characteristic phenotype of osteopetrosis and toothlessness, due to the inability to remodel bone in these mice (6).

A strong association has been found between the overexpression in breast tumor cells of CSF-1 and its receptor, and the progression of breast cancer as well as a poor prognosis (8,9,10,11). Elevated circulating levels of CSF-1 also correlate very strongly with the metastatic process in breast carcinoma (12). CSF-1 expression is prevalent in invasive breast tumor cells as opposed to intra-ductal (pre-invasive) cancer and more cells contain CSF-1 at the invading front than at the center of the tumor mass (10,13). Furthermore, CSF-1 has been shown to stimulate the invasiveness of breast cancer cells in an in vitro amniotic membrane assay (14). The occurrence of tumor-associated macrophages is also highly correlated with the degree of breast tumor angiogenesis related to poor prognosis (15). These data suggest that the macrophage growth factor, CSF-1, induces not only an autocrine tumor cell growth stimulation but also the paracrine recruitment of macrophages into the tumor, emphasizing a potentially important role of tumor associated macrophages.

Besides its potential role in breast carcinogenesis, our previous studies on CSF-1-null mutant mice have shown that CSF-1 plays an important role in mammary gland development since *Csfmop/Csfmop* females display a lack of branching morphogenesis of the mammary gland during pregnancy and fail to undergo lactation (16,17). These defects are associated with an almost complete absence of macrophage in *Csfmop/Csfmop* mammary glands which in normal mice (+/*Csfmop*) cluster around the developing ductal structures at mid-pregnancy.

Our studies supported by this grant have established a relationship between CSF-1-regulated mammary gland development in normal and 0 tumorigenic processes.

## BODY

During this first year, we started to develop different breast tumor mouse models in absence of CSF-1. Early indications suggest that the lack of CSF-1 has significant effect on tumor development. However, during this study, it became apparent that the CSF-1-null mutation affects not only the mammary gland tumor pathologic process but also the normal postnatal mammary gland development. Consequently, we spent a significant time to define the nature of the deficient mammary gland development in young *Csfmop/Csfmop* mice, in order to better understand the impact of CSF-1 on mammary tumorigenesis.

## I – Results

### I.1 – Role of CSF-1 in the etiopathogenesis of mammary gland tumors

The effect of lack of CSF-1 on tumor growth and metastatic potential has been investigated by crossing mice susceptible to mammary tumors with the *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice.

I.1.1 - Task 1: To examine the tumor-suppressive effect of the *Csfm<sup>op</sup>* mutation in the C3H MMTV+ breast tumor susceptible mice

One of these breast tumor susceptibility models is the C3H MMTV+ mouse strain, where breast tumors are induced by the endogenous Mouse Mammary Tumor Virus (MMTV). We backcrossed the *Csfm<sup>op</sup>* mutation 6 times onto the C3H MMTV+ background. The female *+/Csfm<sup>op</sup>* C3H MMTV+ mice have been crossed with the male *Csfm<sup>op</sup>/Csfm<sup>op</sup>* C3H MMTV+ mice, and the *Csfm<sup>op</sup>/Csfm<sup>op</sup>* offspring and their littermate controls have been examined for the incidence of mammary tumor development over their life span. So far, 12 out of 12 *+/Csfm<sup>op</sup>* C3H MMTV+ mice developed mammary tumors in 7 months versus 1 out of 5 *Csfm<sup>op</sup>/Csfm<sup>op</sup>* C3H MMTV+. These preliminary data are in accordance with our hypothesis that in the absence of CSF-1, breast cancer malignancy is reduced.

We also induced surgically breast tumors by transplanting the MM5MT mammary carcinoma cell line (MMTV+, C3H background) into the mammary gland fat pads of mutant mice. We first characterized this cell line in terms of CSF-1 and CSF-1R expression. We showed by northern blot that CSF-1R transcripts are absent and CSF-1 is weakly detected in MM5MT cells. We first attempted to transplant this cell line into the mammary gland fat pads of *Csfm<sup>op</sup>/Csfm<sup>op</sup>* and their littermate control *+/Csfm<sup>op</sup>* mice. Unfortunately, we observed an immunologic rejection of the host; most likely due to the fact that the *Csfm<sup>op</sup>* colony mice lack MMTV and they display a mixed



background C3H/Bl6. Therefore, MMTV and the Bl6 background may be the cause of immunologic rejections. We will next perform the same experiments by transplanting the MM5MT cells into the *Csfm<sup>op</sup>*, C3H, MMTV+ mice that are currently generated. The same C3H, MMTV+ background between the transplanted cells and the mouse will make this tumor mouse model easily interpretable. Unfortunately, we are facing a problem of *Csfm<sup>op</sup>*, C3H, MMTV+ mouse generation, because of their low efficiency of mating. Once we obtain more mice, we hope to achieve this aim.

I.1.2 - Task 2: To establish CSF-1 nullizygous oncogenic-breast cancer mouse models to analyze the role of CSF-1 in breast tumor progression.

In addition to the slow MMTV-infected C3H mammary tumor model, in a parallel study in the laboratory, MMTV-PyV middle T transgenic tumor mouse model has been developed. Mice have been generated by breeding the *Csfm<sup>op</sup>* mutation onto the transgenic MMTV-PyV middle T background. Whole mount analysis of mammary glands from *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice showed a delay in the progression of primary tumors versus the control mice. After 8 weeks, all *+ /Csfm<sup>op</sup>* mice developed tumor multiple foci spread throughout the fat pads, whereas *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice displayed only a primary tumor around the nipple area. Interestingly, the multiple foci in *+ /Csfm<sup>op</sup>* mice are associated with morphologic changes in the main primary tumor around the nipple, displaying an invasive phenotype with a lack of epithelial organization. The degree of tumor malignancy is also associated with an infiltration of leukocytes at the invading front. This delayed tumor progression in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice is correlated to the lack of macrophage, which are abundant around the carcinoma cells in *+ /Csfm<sup>op</sup>* mice.

A third tumor model *neu/cerb2* is still in progress. We are currently breeding the transgenic MMTV-*neu/cerb2* mice onto the *Csfm<sup>op</sup>/Csfm<sup>op</sup>* background.

However, there is a caveat in the interpretation of these results, since we found that the mammary gland development in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice was delayed compared with *+ /Csfm<sup>op</sup>* mice, suggesting that this rather than the involvement of macrophages in tumor growth might be the explanation of the difference between *+ /Csfm<sup>op</sup>* and *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice. It became essential therefore, to define the developmental deficiencies and the reason for these defects.

## **I.2 - Role of CSF-1 in prepubertal mammary gland development**

### **I.2.1 - Requirement of CSF-1 in mammary gland development**

We studied the development of *+ /Csfm<sup>op</sup>* and *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice's mammary glands from 3 to 12 weeks of age (figure 1). The fourth inguinal mammary gland whole mounts of 3 to 5 mice were analyzed and the different parameters such as duct length, branching number, terminal end bud

(TEB) number were quantified (figure 2). Ductal lengths (mm) were measured from the nipple area to the tip of the longest duct through the lymph node. Branching numbers represent the mean of branching number along the 3 longest ducts from the nipple area to the migration front. TEBs were quantified in the whole mammary gland. The first TEBs, which are the initial epithelial structure for duct elongation, appear at 3 weeks in mammary gland of WT mice (+/*Csfm<sup>op</sup>*) compared to 4 weeks in the mutant mice (*Csfm<sup>op</sup>/Csfm<sup>op</sup>*). In addition, the TEB number in WT mammary gland is significantly greater than in mutant mammary gland, which is closely related to the more extensive ductal tree and the larger fat pad size in mammary gland of +/*Csfm<sup>op</sup>* mice. Between 8 and 9 weeks, the branching tree filled the whole fat pad in +/*Csfm<sup>op</sup>* mice. TEB have disappeared and some secondary ducts, resulting from the hormonal estrus cycle influence, are formed. The ductal tree is still growing in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice and finally, at 12 weeks of age, the branching tree filled the fat pad. However, no secondary ducts are noticed, most likely due to the delayed estrus cycle in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice. Interestingly, the parameters describing the mutant aberrant mammary gland development such as, duct length, branching and TEB numbers, do not follow the difference of mouse weight observed from the very beginning of mouse life (3 weeks of age, figure 2), indicating that the mammary gland phenotype in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice is not due to a general metabolism defect, but rather due to a local mammary gland defect.

Taken together, these data show a delayed mammary gland development in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice in addition to an atrophic adult mammary gland formation. The delay in development paralleled the delay in tumor progression suggests that there is a common mechanism between these processes.

#### I.2.2 - Requirement of macrophage in TEB and branching formation.

Since CSF-1 is the major growth factor involved in mononuclear phagocyte proliferation, differentiation and recruitment, we addressed the question whether the defective ductal tree in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice was due to a macrophage population deficiency. We analyzed macrophage distribution during prepubertal mammary gland development in the CSF-1 null mutant mice and their littermate controls, by performing immuno-histochemistry with the antibody F4/80 specific for macrophages. However, our data indicate clearly that F4/80 is not only able to recognize macrophages but also cross react with eosinophils in mammary gland. All eosinophils identified by a giemsa staining surrounding TEBs, are positive for F4/80. Among F4/80 positive cells, eosinophils are easily distinguished from macrophages at high power magnification (100X) by their nuclear shape (polynuclear for eosinophil and mononuclear for macrophage) and their cytoplasmic shape (usually round for eosinophil and largely spread for macrophage). Very early in the development of the mammary gland, at 2.5 weeks of age, macrophages are already abundant around the nipple area in +/*Csfm<sup>op</sup>* mice compared to the *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice, where macrophages are completely absent. Then, at 3 weeks of age, when TEBs appear in +/*Csfm<sup>op</sup>* mice, macrophages and eosinophils are

recruited around all TEBs, suggesting different functions in ductal outgrowth. In contrast, in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice, no F4/80 positive cells were found around the rudimentary tips of ducts, that at this time have not yet formed TEB. Most of F4/80 positive cells present in the mammary gland come from the lymph node leading to an intense F4/80 immunostaining into the lymph node of both *Csfm<sup>op</sup>/Csfm<sup>op</sup>* and *+ /Csfm<sup>op</sup>* mice. In the control mice, the F4/80 positive cells that are released in the fat pad from the lymph node are equally distributed between macrophage and eosinophil (figure 4). In contrast, in the CSF-1 null mutant mice, eosinophils are the only type of F4/80 positive cells seen and they are abundantly scattered all over the fat pad. These data, so far, indicate that macrophage recruitment is closely related to TEB formation. At 5 weeks of age, F4/80 positive cells are recruited around the TEBs that are developed in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice. Half of them are macrophages, and half are eosinophils. However, the F4/80 positive cell density surrounding TEBs is about half in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice versus control mice (figure 4).

### I.2.3 - Daily CSF-1 treatment from birth rescues partially the mammary gland defect related to the lack of CSF-1-dependant macrophage recruitment in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice.

In order to determine whether CSF-1 is indispensable for ductal outgrowth, we treated the mutant mice and their littermate control mice from birth with a daily injection of CSF-1. We examined the potential rescue of the defective mammary gland development in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice. Mice were sacrificed at 5 weeks of age, and mammary gland whole mounts were analyzed. Interestingly, CSF-1 treatment rescued the branching number and TEB number in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice, highlighting the important role of CSF-1 in branching outgrowth. However, the ductal length remained low compared to the non-treated control mice (Figure 3). Similarly, the fat pad size was still reduced in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice, suggesting a closed correlation between the control of fatty stroma size and ductal length. CSF-1 treatment did not change significantly the weight of mice in both group's *+ /Csfm<sup>op</sup>* and *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice, which is consistent with the fact that mammary gland fat size did not change in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice.

After 5 weeks of daily CSF-1 treatment, F4/80 positive cells density around TEBs is not significantly different in *+ /Csfm<sup>op</sup>* mice versus the mutant mice and did not increase compared to the untreated mice in both groups (figure 4). Nevertheless, most of these F4/80 positive cells are macrophages, the number of eosinophil having strongly decreased. The northern blot analysis of total mammary gland RNA from 5 weeks of age mice shows a strong CSF-1R transcript level in *+ /Csfm<sup>op</sup>* mice, while it is almost undetectable in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice. CSF-1R transcript level is rescued in mammary glands of *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice treated with CSF-1 from birth, indicating that CSF-1 itself upregulates its receptor CSF-1R transcript in mammary gland. Preliminary immunohistochemistry data showed that in *+ /Csfm<sup>op</sup>* mice, CSF-1R protein is expressed in epithelial cells from the TEB bodies and from the luminal side of ducts as well as in the macrophages especially localized in lymph node and in the connective tissue surrounding the fatty stroma. CSF-

1R immunostaining is not detectable in the mammary gland of *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice. Altogether these data show a strong evidence of CSF-1 requirement in early mammary gland development, acting through its receptor CSF-1R directly on epithelial cells and indirectly on macrophages by recruiting them in the mammary gland from the lymph node and the adjacent connective tissue.

The tight correlation between the dramatic recruitment of CSF-1-dependant macrophages around TEBs and ductal growth highlights the importance of CSF-1 in postnatal mammary gland development acting through the macrophage/epithelial cell interactions. This developmental study is also consistent with the role of macrophages in tumor progression and suggests that macrophages promote the ability of both ductal epithelial and tumor cells to invade the fat pad. Nevertheless, the unexpected finding of CSF-1R immunoreactivity in ductal epithelial cells suggests that CSF-1 could have a direct role in ductal epithelial and tumor growth. These alternatives will be explored during the rest of the year of funding.

#### I.2.4 – Role of CSF-1 in the in the adipocyte/epithelial cell interaction in mammary gland development.

In order to determine the crucial time when the deficiency of mammary gland epithelial growth in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice is observed, we analyzed mammary gland whole mounts very early during the development (figure 5). Surprisingly, at 2 weeks of age, the mammary glands of mutant and WT mice display a similar development. Branching trees remain very rudimentary without any TEB and fat pad sizes are identical. In contrast, at 3 weeks of age, the first TEB are formed in *+Csfm<sup>op</sup>* mice whereas they are still absent in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice. This defective epithelial growth is most importantly associated with a dramatic reduction of the fat pad size in mutant mice. Therefore, we focussed our studies on the role of CSF-1 on fat pad development. We performed subtraction hybridization between an mRNA pool derived from 3 weeks mammary glands of *+Csfm<sup>op</sup>* mice and an mRNA pool derived from 4 weeks mammary glands of *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice. At these ages, *Csfm<sup>op</sup>/Csfm<sup>op</sup>* and *+Csfm<sup>op</sup>* mice display the same epithelial tree outgrowth with some TEBs. The only morphologic difference resides in the fat pad size, which is strongly reduced in the mutant mice (figure 5). Therefore, by subtraction hybridization, we sought to identify genes that are specifically associated with CSF-1-dependant signaling in fat exclusive of genes involved in TEB formation. Indeed, we found one such gene, the uncoupling protein -1 (UCP-1), which is known to be specifically expressed in brown fat adipocytes (18). The northern blot of mammary gland total RNA from different mice of 2 to 4 weeks of age showed the presence of UCP-1 transcript exclusively in *+Csfm<sup>op</sup>* mice. The histologic analysis of mammary glands of *+Csfm<sup>op</sup>* mice at this early stage confirmed the presence of multilocular adipocyte foci characteristic to brown fat tissue (figure 6). The brown fat is localized between the nipple and the lymph node, in the area where TEBs are formed. Our data indicate a close relationship between the brown fat presence and TEB development in *+Csfm<sup>op</sup>* mice. This is the first report indicating the presence of brown fat in mammary gland

suggesting a novel role of this specific fatty stroma in epithelial mammary gland outgrowth. Nevertheless, the direct role of CSF-1 for adipocyte differentiation remains to be elucidated, and the contribution of the brown fat for TEB formation must be further investigated.

In conclusion, this developmental study of CSF-1 null mutant mice allows us to demonstrate multiple role of CSF-1 in postnatal mammary gland development. CSF-1 is required for epithelial ductal outgrowth acting through the CSF-1R expressed directly on epithelial cells, and indirectly on macrophages in order to recruit them around the highly proliferative epithelial structure, the TEBs. Although the expression of CSF-1R on brown adipocytes is not definitive, our preliminary data suggest that CSF-1 regulates the transient appearance of brown fat cells in the mammary gland closely associated with TEB formation.

## II – Discussion

Our original hypothesis was that CSF-1 is involved in the breast tumor progression acting in an autocrine manner on tumor epithelial cells but also in a paracrine manner on CSF-1R bearing macrophages recruitment into the tumor. Therefore, in absence of CSF-1, breast cancer malignancy should be strongly reduced.

During this first year, we showed that, in absence of CSF-1 (*Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice), the mammary gland tumor incidence was significantly reduced in the MMTV infected C3H mouse model. In addition, by analyzing a more aggressive mouse model, the transgenic MMTV-PyV middle T background, we found a delayed tumor progression in *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice correlated to the lack of macrophage, which are abundant around the carcinoma cells in *+/Csfm<sup>op</sup>* mice. So far, our data are consistent with our original hypothesis indicating the crucial role of CSF-1 and CSF-1-dependant macrophages in tumor cell growth and invasiveness. Similarly, in a normal mammary gland development context, we demonstrated that CSF-1 is required for proper postnatal mammary gland development acting through CSF-1R-bearing-macrophages and -epithelial cells. In addition to its influence on macrophages, CSF-1 is also able to affect adipocyte differentiation into brown fat cells, which is associated with the branch outgrowth during the postnatal mammary gland development. Interestingly, tumor development correlated with reduced mammary gland development most likely due to effect on fat pad. In deed, well-documented mesenchymal /epithelial interactions suggest that this point is important both in normal as well as malignant mammary epithelium growth. These studies, altogether with ongoing analysis of mammary tumor-susceptible *Csfm<sup>op</sup>/Csfm<sup>op</sup>* mice, will offer insight into the autocrine role of tumor-secreted CSF-1 and its paracrine effect on macrophages and fat cells, emphasizing the importance of stromal/epithelial cell interactions in normal and malignant mammary gland development.

## CONCLUSION

We showed that in addition to the direct role of CSF-1 on epithelial cell growth, CSF-1 orchestrates the dense stroma (macrophage)/ epithelial cell interactions as well as the fatty stroma (brown adipocyte)/epithelial cell interactions necessary for proper mammary gland epithelial development. These results are consistent with the tight correlation we found between tumor-macrophage infiltration and the degree of mammary tumor malignancy. This new concept of the multiple action of CSF-1 in mammary gland postnatal development provides a new approach to investigate CSF-1 function in breast tumor progression focussed on macrophage and brown adipocyte roles. Consequently, beside our original proposal, we plan to investigate further the novel observation of CSF-1 function on brown fat differentiation leading to the postnatal mammary gland development and potentially to the breast tumor malignancy.

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## APPENDICES

**Figure 1 :** Mammary glands whole mounts of  $+/Csfn^{op}$  and  $Csfn^{op}/Csfn^{op}$  virgin mice of 3, 4, 9 and 12 weeks of age. The photomicrographs were taken at the same magnification of the entire fourth inguinal mammary gland, showing the atrophic development in  $Csfn^{op}/Csfn^{op}$  mice. The arrow indicates the terminal end buds (TEB), NP is the nipple area and LN the lymph node.

**Figure 2 :** Ductal length, branching and terminal end bud (TEB) numbers and weight of  $+/Csfn^{op}$  and  $Csfn^{op}/Csfn^{op}$  mice. Mice were killed at 2.5 to 12 weeks, and the fourth inguinal mammary gland whole mounts were analyzed. The ductal length (mm) is measured from the nipple area to the tip of the longest duct through the lymph node. Branching number is the mean of branching number along the 3 longest ducts from the nipple area. TEBs are quantified in the whole mammary gland. Points represent mean  $\pm$  SD of at least three mice per time point.

**Figure 3 :** Ductal length, branching and TEB numbers of  $+/Csfn^{op}$  and  $Csfn^{op}/Csfn^{op}$  mice. Mice treated with CSF-1 from birth and their untreated littermates were killed at 5 weeks, and the fourth inguinal mammary gland whole mounts were analyzed. The ductal length (mm) is measured from the nipple area to the tip of the longest duct through the lymph node. Branching number is the mean of branching number along the 3 longest ducts from the nipple area. TEB are quantified in the whole mammary gland. Points represent mean  $\pm$  SD of at least three mice per time point.

**Figure 4 :** Quantification of F4/80 positive cells per unit surface localized around TEBs in  $+/Csfn^{op}$  and  $Csfn^{op}/Csfn^{op}$  mice. Mice treated with CSF-1 from birth and their untreated littermates were killed at 3 or 5 weeks, and F4/80 immunostaining of each fourth inguinal mammary gland were analyzed. Macrophage and eosinophil were distinguished and counted for 3 TEBs on the mammary gland section from 3 mice of each group. Points represent mean  $\pm$  SD.

**Figure 5 :** Mammary gland whole mounts of  $+/Csfn^{op}$  and  $Csfn^{op}/Csfn^{op}$  mice of 2 and 3 weeks of age. The photomicrographs were taken at the same magnification of the entire fourth inguinal mammary gland, showing the reduced fat pad size in  $Csfn^{op}/Csfn^{op}$  mice at 3 weeks.

**Figure 6 :** Mammary gland sections of  $+/Csfn^{op}$  and  $Csfn^{op}/Csfn^{op}$  mice of 2.5 weeks of age are stained with hematoxylin and eosin. The typical multilocular brown adipocytes are abundantly present in mammary gland of  $Csfn^{op}/Csfn^{op}$  mice.

Figure 1

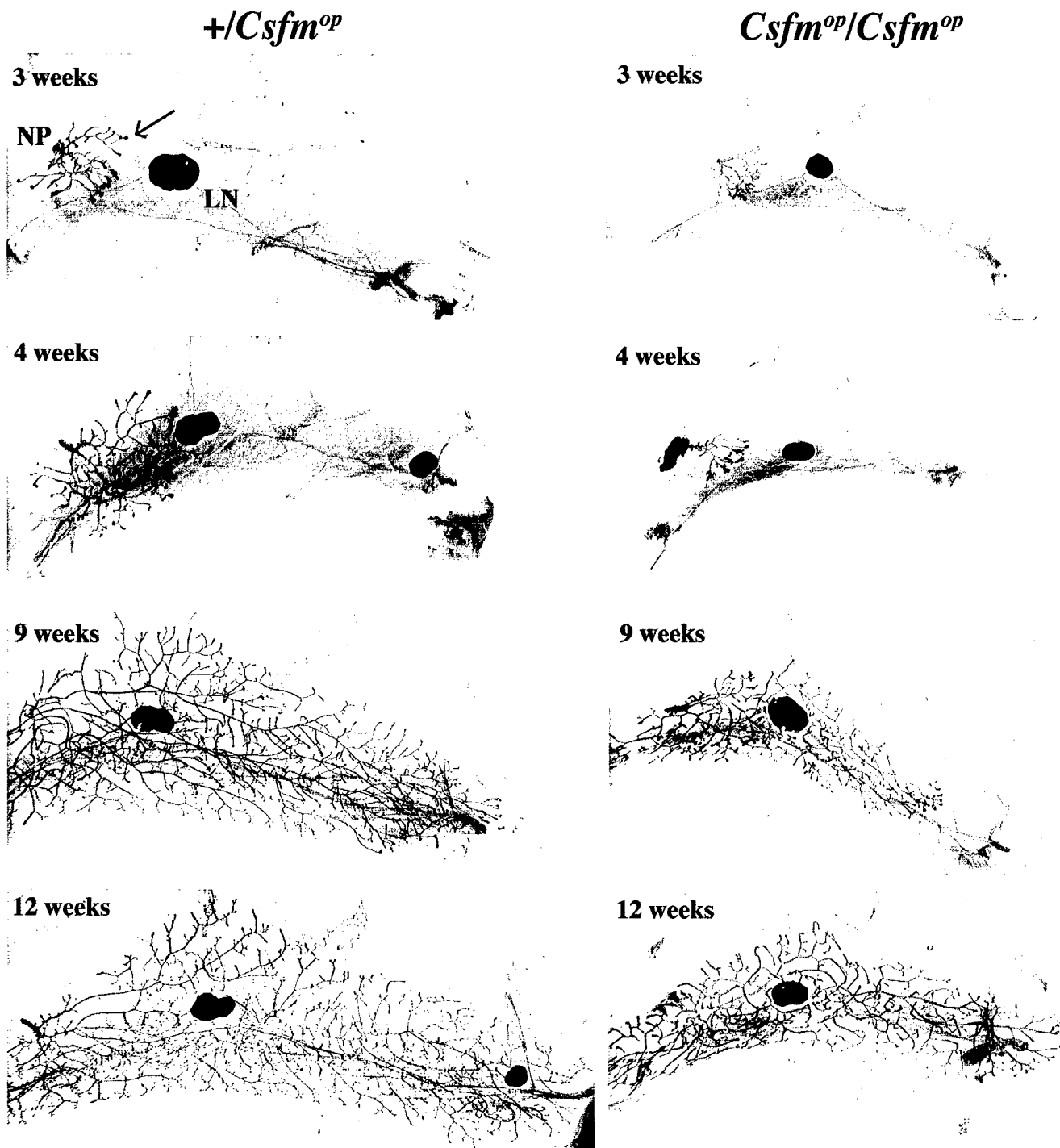


Figure 2

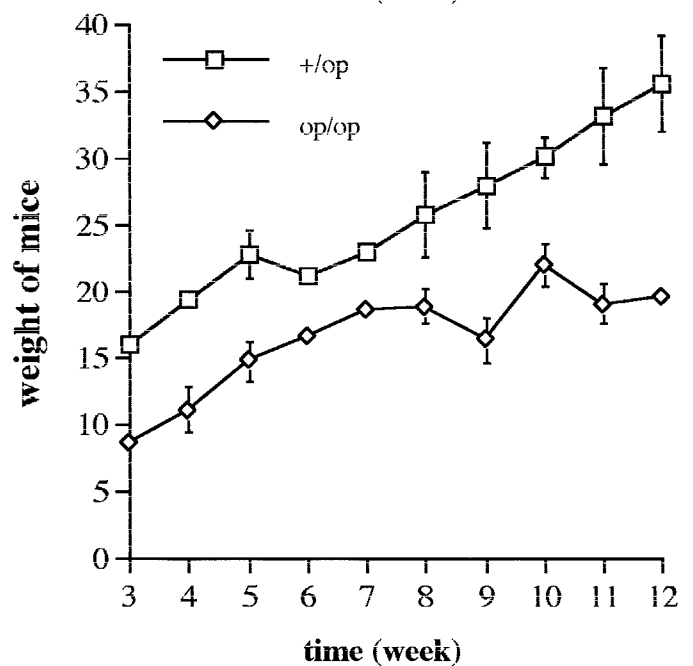
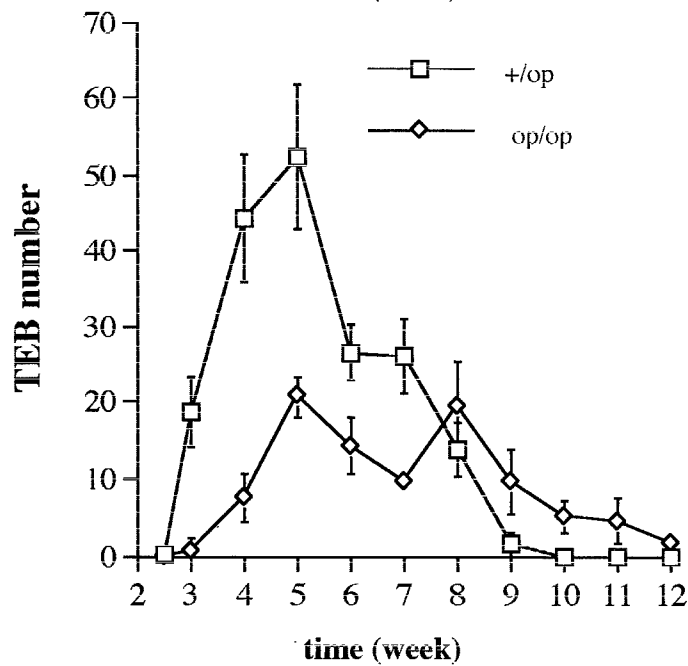
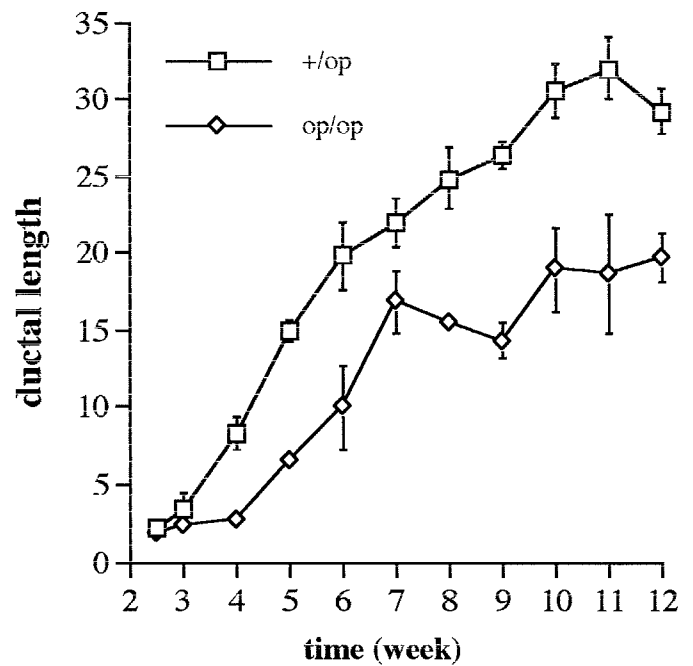
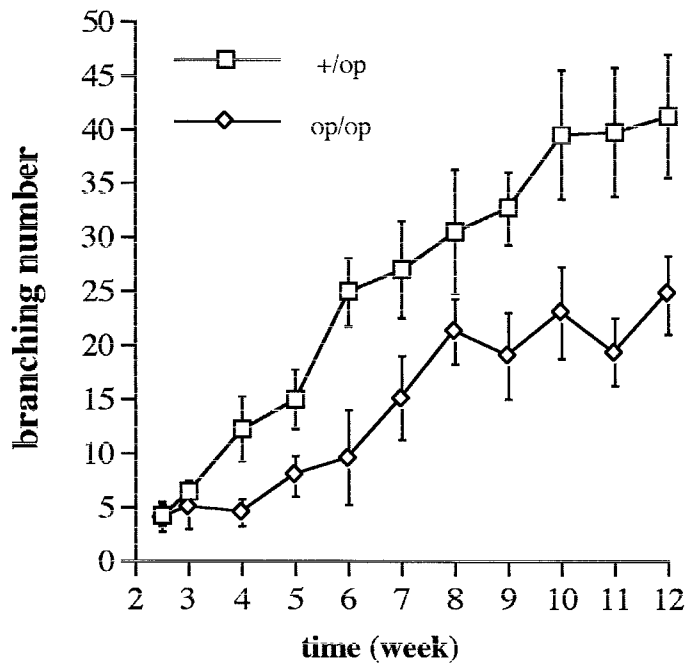


Figure 3

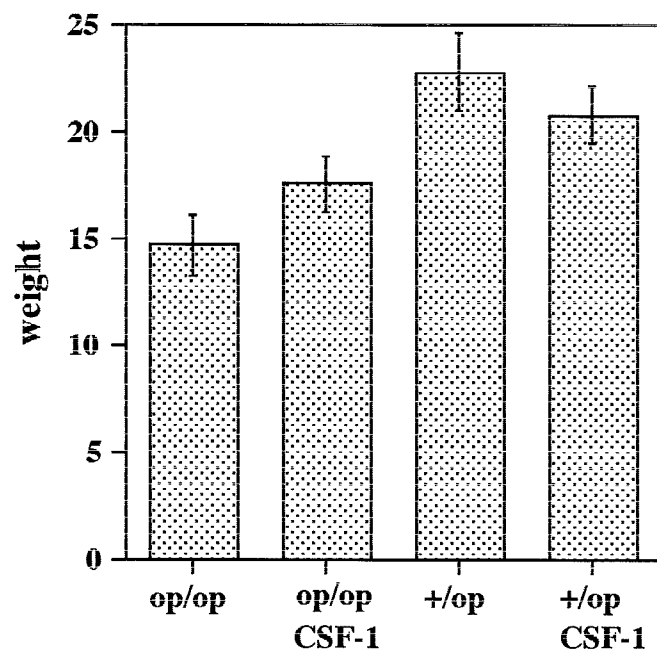
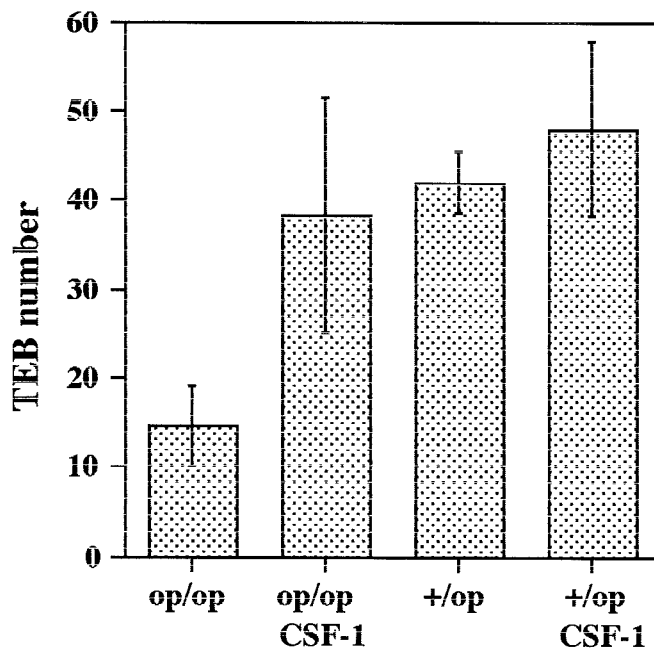
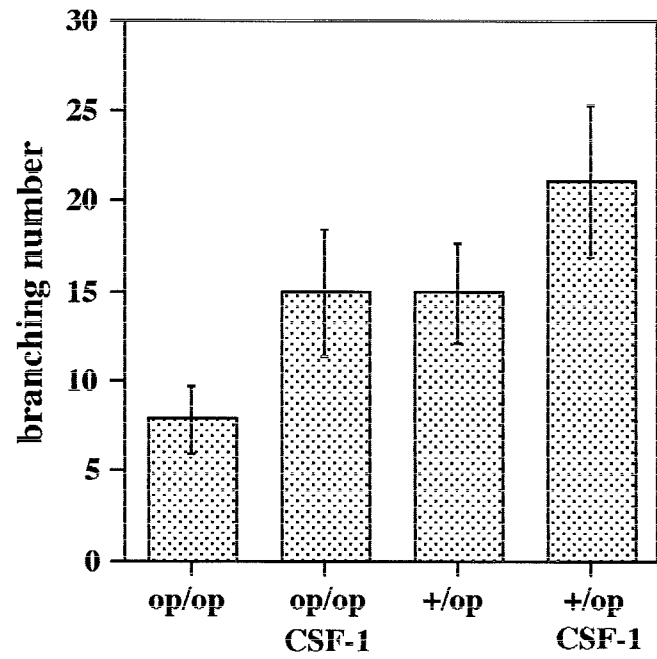
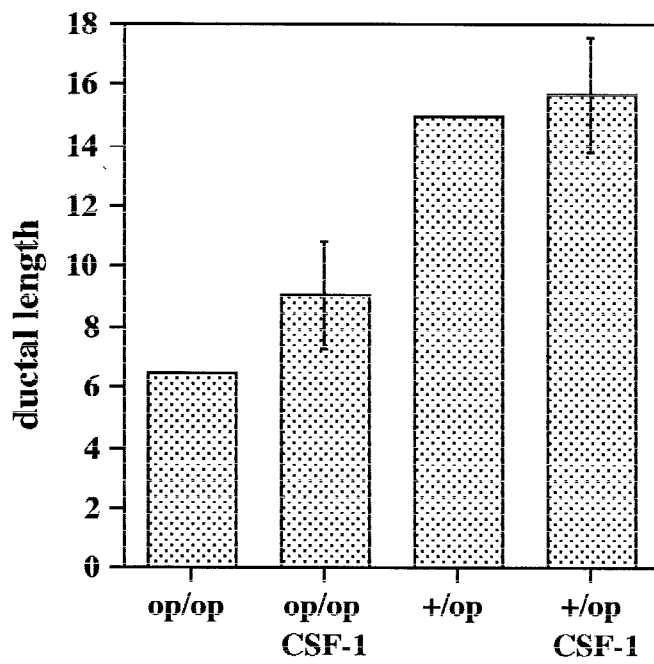


Figure 4

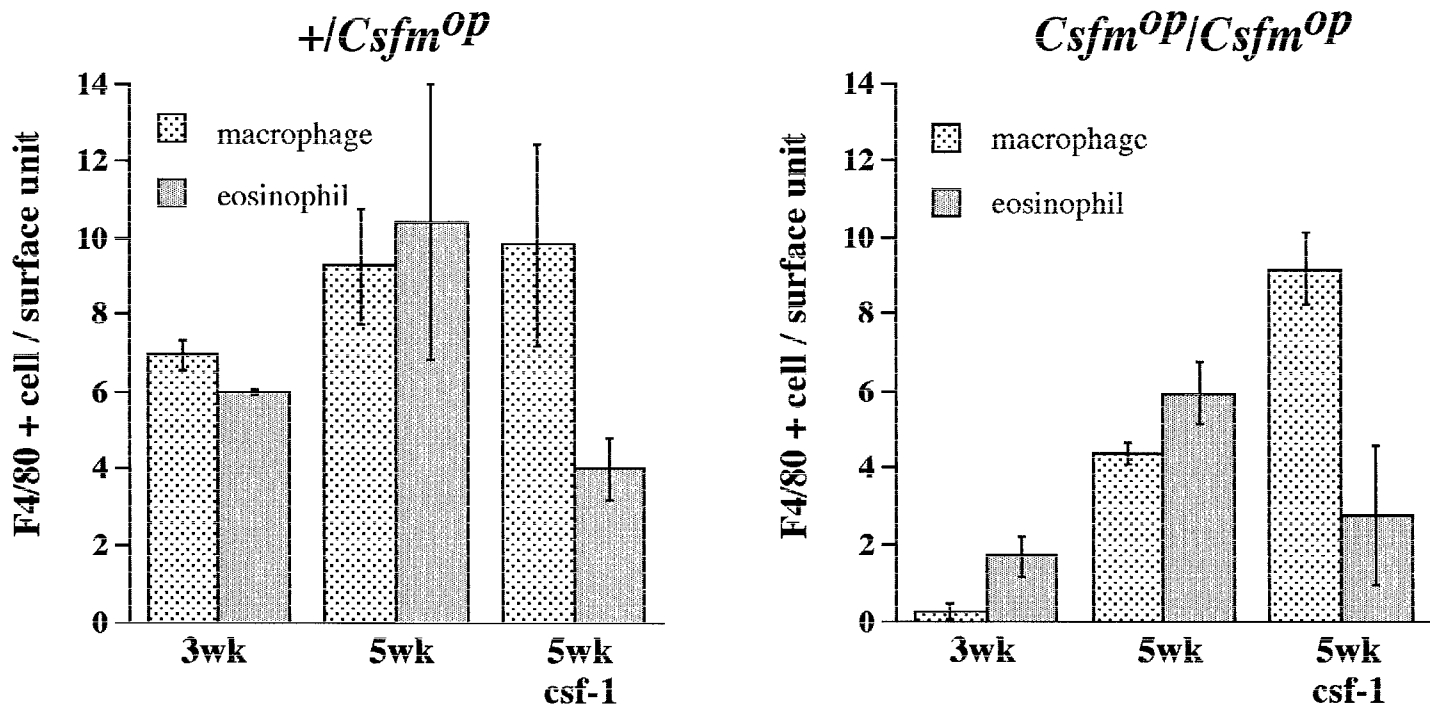


Figure 5

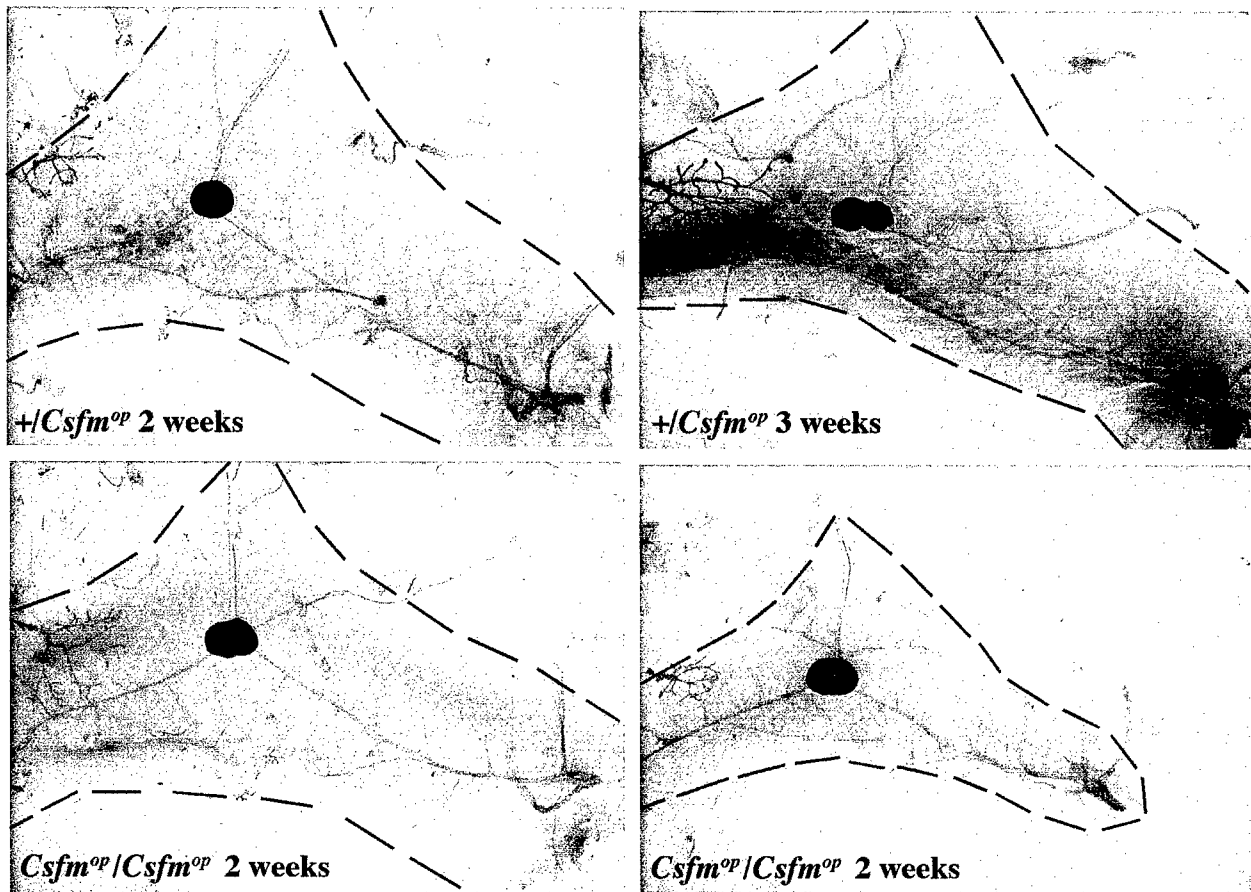
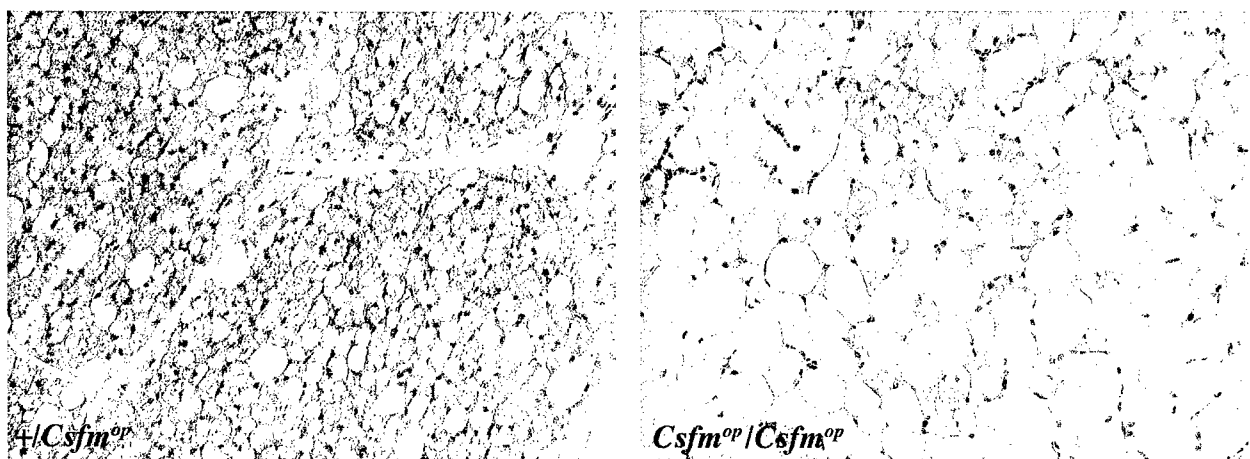


Figure 6





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